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Identification of a Naturally Occurring Recombinant Epstein-Barr Virus Isolate from New Guinea That Encodes both Type 1 and Type 2 Nuclear Antigen Sequences

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In this report we describe an Epstein-Barr virus isolate, derived from the peripheral blood lymphocytes of a healthy adult from Papua New Guinea, that is a recombinant of the two major Epstein-Barr virus types, encoding type 1 Epstein-Barr nuclear antigen 2 (EBNA2) sequences, and type 2 EBNA3, EBNA4, and EBNA6 sequences.

Epstein-Barr virus (EBV), a lymphotropic herpesvirus that also infects and replicates in oropharyngeal epithelial cells, has a long-established association with *Homo sapiens*. It is the causative agent of acute infectious mononucleosis and an acute lymphoproliferative disease in immunosuppressed patients and is associated with Burkitt's lymphoma and nasopharyngeal carcinoma (16). The multitude of different EBV strains can be classified into two broad types designated EBV-1 and EBV-2, paralleling the nomenclature of herpes simplex virus types 1 and 2. The two EBV types (which are also referred to as A type and B type) are distinguished by divergent DNA sequences in genes that encode several of the EBV nuclear antigens (EBNAs). EBNA3, EBNA4, EBNA6 (also referred to as EBNA3A, EBNA3B, and EBNA3C, respectively), and EBNA2 of the prototype EBV-1 strain (derived from the B95-8 cell line) differ in their amino acid sequences from corresponding proteins of the prototype EBV-2 strain (derived from the Ag876 cell line) by 16, 20, 28, and 47%, respectively (1, 10, 20). These differences are reflected in the common detection of antibody (23, 32, 33) and cytotoxic T lymphocyte (7, 8, 17) responses that are EBV type specific. While most individuals in Western societies carry EBV-1, a substantial proportion of the normal populations of Africa and New Guinea is infected with EBV-2 (32, 33). Coinfection with EBV-1 and EBV-2 has been demonstrated in Caucasian individuals (13, 23, 30) and is particularly common in immunosuppressed patients, in which individuals the susceptibility to superinfection is presumably higher (5, 13, 21, 26).

This report describes the unusual EBV isolate MK, which was derived from a lymphoblastoid cell line (LCL) raised from a healthy adult male born in Chimbu Province in the highlands of Papua New Guinea. The LCL was established by spontaneous outgrowth from peripheral lymphocytes cultured in the presence of 0.1 μg of cyclosporin A per ml (18). DNA samples from the MK LCL were always derived from early passages of the cell line. To determine the EBV type of this isolate, PCR was used to amplify a region of the EBNA2 gene with primers specific for either EBV-1 (2A.1 and 2A.2, based on the B95-8 sequence) or EBV-2 (2B.1 and 2B.2, based on the Ag876 sequence) (2). The PCR was carried out as previously described (2), and amplified products were electrophoresed on a 2% agarose gel (SeaKem; FMC Bioproducts). The products obtained with the 2A.1 and 2A.2 or 2B.1 and 2B.2 primers were confirmed to be of type 1 or type 2 origin, respectively, by testing duplicate samples by a rapid PCR enzyme-linked immunosorbent assay (ELISA) method (Boehringer, Mannheim, Germany) and with the capture probes ^{49,245}5′CATCTCCCTG TCTTGCATGTGCCAGACCAA3′^{49,274} (B95-8 coordinates, type 1 specific) and ^{2,107}5′CTAATCAACCAGCCACAACAC CACCCACGG3′^{2,136} (Ag876 coordinates, type 2 specific) (data not shown). Briefly, this involved labelling the PCR products with digoxigenin during the amplification process, after which a solution hybridization colorimetric analysis was performed with the specific biotin-labelled capture probes, an anti-digoxigenin peroxidase conjugate, and the substrate 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS). As shown in Fig. 1A and B, the New Guinea isolate MK (lanes 4) was defined as type 1 with these EBNA2 primers. Other samples included the control type 1 B95-8 strain (Fig. 1A and B, lanes 2) and the type 2 Ag876 strain (lanes 3), as well as four other isolates derived from LCLs raised spontaneously from healthy New Guinea donors which were also defined as type 1 (lanes 5 to 8).

In a recent study designed to analyze sequence variation within wild-type EBV-1 isolates from New Guinea at regions of the nuclear antigens corresponding to defined cytotoxic T lymphocyte epitopes (6), the EBV isolate MK could not be sequenced within an area of EBNA3 because of the inability to amplify extracted DNA with primers specific for type 1 EBV. To further investigate the EBNA3 gene of this isolate, viral DNA was typed by the above procedure but with primers from another EBNA3 region that were also specific for type 1 EBV. These primer sequences, with B95-8 coordinates, were ^{93,682}5′TAGCAGCTCAGGGAATGGCA3′^{93,701} (E3A.F) and ^{93,993}5′GAACTGGCTTGGGGTAAACT3′^{93,974} (E3A.R). The rapid PCR ELISA method, with the EBNA3 type 1-specific probe ^{93,758}5′TACGCCCTTGCCCCCTGTATCTCCAG3′^{93,783}, was used to confirm that the products obtained were of type 1 origin (data not shown). Surprisingly, DNA from the MK EBV isolate (Fig. 1C, lane 4) was not amplified with these primers,

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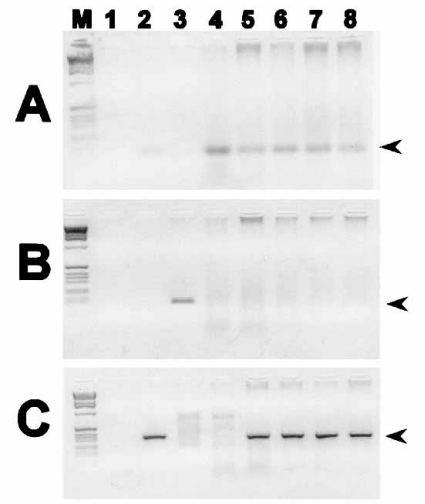

FIG. 1. Typing of EBV present in New Guinea spontaneous LCLs by PCR amplification. DNA was extracted from LCLs and subjected to PCR amplification with primers complementary to regions within the type 1 EBNA2 (A), type 2 EBNA2 (B), and type 1 EBNA3 (C) genes. PCR mixtures were separated by electrophoresis and loaded as follows: lanes M, 1-kb molecular weight markers; lanes 1, water control; lanes 2, B95-8; lanes 3, Ag876; lanes 4, MK; lanes 5, H22; lanes 6, H9; lanes 7, L10; and lanes 8, L13. Arrowheads indicate amplified products.

although PCR products were observed with the B95-8 strain (lane 2) and the other New Guinea isolates (lanes 5 to 8) but not with the EBV-2 strain Ag876 (lane 3). The MK isolate therefore appeared not to be type 1 with these primers.

To determine if these apparently contradictory results were simply a reflection of minor interstrain sequence divergence or perhaps an EBNA3 deletion, areas of the EBNA3, EBNA4, and EBNA6 genes of the MK isolate were sequenced with the primers listed in Table 1. PCR products were purified with QIAquick spin columns (Qiagen Inc., Chatsworth, Calif.) and sequenced in both directions with a PRISM ready reaction dyedeoxy terminator cycle sequencing kit and a 373A DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) by following the manufacturer's protocol. Two EBNA3 regions totalling 218 nucleotides in length (22 of which differ between the B95-8 and Ag876 strains) were sequenced in MK and found to be identical in all but two positions with the EBV-2 strain Ag876 (Table 1). In addition, the MK isolate was sequenced over a 472-nucleotide section of EBNA4 and a 248-nucleotide section of EBNA6, where the B95-8 and Ag876 sequences vary by 72 and 36 nucleotides, respectively. The MK and Ag876 isolates were found to be identical within these regions except for two nucleotide mismatches within the EBNA4 region and one nucleotide mismatch within the EBNA6 region (Table 1). These EBNA3, EBNA4, and EBNA6 regions of the MK isolate can therefore be classified as type 2.

A region of the EBNA2 gene of the MK EBV isolate was then sequenced over an area where type 1 and type 2 EBV are highly divergent, corresponding to the B95-8 EBV coordinates 48810 to 49411. Also included in this sequence analysis were multiple New Guinea (highland and coastal) and Australian Caucasian EBV-1 isolates. These were derived from spontaneous LCLs established from blood samples collected from healthy donors from the Madang Province (coastal) and the Eastern Highlands Province of Papua New Guinea (32) and from Brisbane, Australia. Previously described primers (2) were used to amplify the region, and two additional primers, ^{49,148}5′AAGGCCTACCCGTCCTACCA3′^{49,167} and ^{49,061}5′CGGTTGTGACAGAGGTGACA3′^{49,042}, were used to assist in sequencing. As shown in Fig. 2, the MK EBV isolate and the nine EBV-1 isolates from New Guinea have the common feature of a 51-bp deletion between nucleotides 49089 and 49141 relative to the B95-8 strain. This deletion was also described by Aitken et al. for two EBV-1 isolates from New Guinea but was not seen in three African EBV-1 isolates or in four EBV-2 isolates (from Africa and New Guinea) previously sequenced in this EBNA2 region (2, 9). The deletion was also not found in five EBV-1 isolates from Australian Caucasians (data not shown). A previous study of laboratory-generated deletion mutants has also demonstrated that this deleted region of EBNA2 is nonessential for EBV transformation of B lymphocytes (9).

Excluding the deletions, the overall number of nucleotide mismatches within this EBNA2 region between the MK and the B95-8 strains is 11 (8 amino acids), and between MK and the prototype EBV-2 strain, Ag876, the number of mismatches

TABLE 1. Nucleotide differences within regions of EBNA3, EBNA4, and EBNA6 of the MK EBV isolate compared with the B95-8 and the Ag876 strains

Gene	Region sequenced[a]	No. of mismatches of MK isolate with:		Position of Ag876 mismatch[a]	Nucleotide at indicated position of isolate:		Primers used for PCR and/or sequencing
		B95-8	Ag876		Ag876	MK	
EBNA3	723–840	13	0				^{92,736}5′ATGTATGCCATGGCCATTCGA3′^{92,756b} ^{92,917}5′AGATGTACGAATGTGGGAGTC3′^{92,897b}
EBNA3	1193–1292	11	2	1239	C	T	^{93,226}5′TGGGCACCACTAGTATCCAA3′^{93,245b}
				1248	C	A	^{93,378}5′TTTCACCGGTAGCACCTTCG3′^{93,359b}
EBNA4	4398–4869	73	2	4455	G	A	^{96,494}5′TTGTTGAGGATGACGACG3′^{96,511b}
				4727	C	T	^{97,005}5′CAGTAGGGTTGCCATAAC3′^{96,988b}
							^{96,834}5′ACTGAGTGTCCAGGCTCG3′^{96,851b}
							^{96,722}5′CTGTGTTCCTCTTCAATG3′^{96,705b}
EBNA6	7639–7886	35	1	7765	G	A	^{7,635}5′GTGAAGCGCACAATTGTTAA3′^{7,654a} ^{7,890}5′CCTCTATGATATCATCGTCA3′^{7,871a}

[a] Numbering of these nucleotide sequences is consistent with that used for the Ag876 EBV strain by Sample et al. (20).
[b] These primers and their coordinates are based on the B95-8 strain (3), but PCR amplify both EBV-1 and EBV-2 DNA.

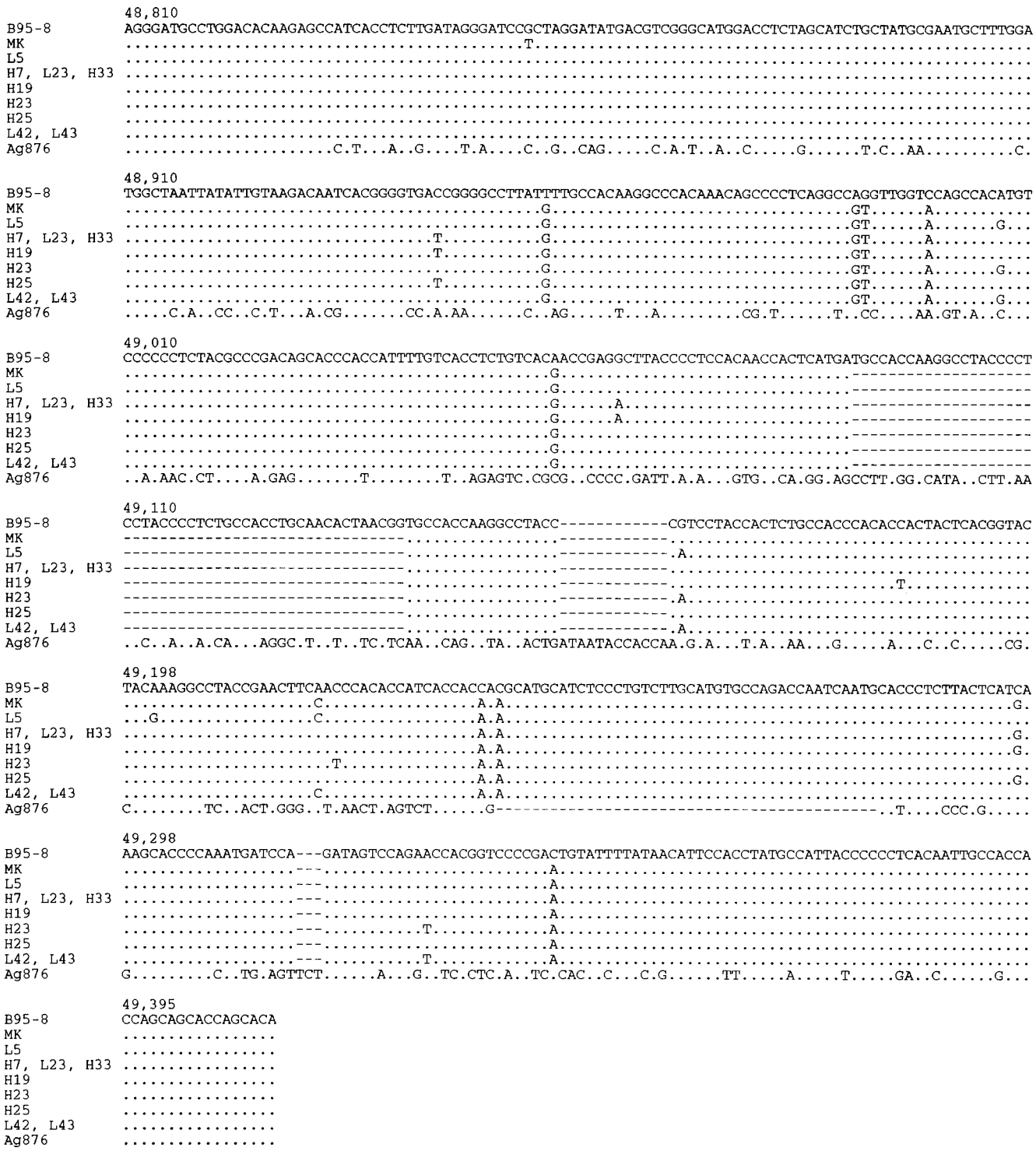

FIG. 2. EBNA2 DNA sequences, corresponding to the B95-8 EBV coordinates 48810 to 49411 (3), of 10 EBV isolates from New Guinea compared with the prototype EBV-1 strain from B95-8. Differences with the B95-8 strain are indicated immediately below the corresponding B95-8 nucleotides. A hyphen indicates a deleted base, and a dot signifies nucleotide identity. Also shown is the sequence of the prototype EBV-2 strain from Ag876 (10). New Guinea isolate names beginning with the letter H or L are from the highland or coastal areas, respectively.

is 178 (Fig. 2). In contrast, the New Guinea EBV-2 isolates L4 and L19 are mismatched in this region by just 3 and 4 nucleotides, respectively, with the Ag876 strain (2). The MK EBV isolate can therefore be classified as type 1 in this EBNA2 region. Eight of the 11 sequence mismatches between the MK and the B95-8 strains are shared by all the other EBV-1 isolates from New Guinea (Fig. 2). Four of these (A → G, G → T, C → A, and A → G at positions 48990, 48991, 48998, and

49057, respectively) are also present in the three previously sequenced African EBV-1 isolates (2, 9), in three of the five EBV-1 Caucasian isolates included in our analysis (data not shown), and in an EBV-1 isolate from an oral hairy leukoplakia biopsy specimen (28). The sequence of the B95-8 strain may therefore be atypical at these positions. There are also three nucleotides (G, A, and A at positions 48956, 49237, and 49342, respectively) that, together with the 51-bp deletion, appear to be characteristic of New Guinea EBV-1 isolates, being present in all isolates from both highland and coastal areas and not in the isolates from Australian Caucasians (data not shown) or in other isolates previously sequenced in this region (2, 9, 28). These data are therefore consistent with our recent report, based on EBNA3 and EBNA4 gene sequence analysis (6), suggesting that the EBV-1 strains of both highland and coastal areas of New Guinea have evolved along a single distinct lineage, displaying common sequence differences from strains found in other parts of the world. The sequence homology between the New Guinea EBV-1 isolates is surprising given the human genetic diversity in the country and current theories that the highland and coastal areas were settled by separate waves of migration occurring many thousands of years apart (4, 22).

This report has identified an EBV isolate (MK) that is a hybrid of the two major EBV types, being type 1 in a region of EBNA2 and type 2 in regions of EBNA3, EBNA4, and EBNA6. In addition to the samples used for Fig. 1, 22 other EBV isolates from New Guinea and 5 from Caucasian Australians were also tested with the type-specific EBNA2 and EBNA3 primers, but no other similar EBV-1/EBV-2 hybrid strains were found (data not shown). Interestingly, however, similar EBV hybrids have recently been isolated in another laboratory from individuals with concurrent human immunodeficiency virus infection (31).

How could such hybrids have arisen? Recent studies have demonstrated that EBNA2 is an exceptionally active region for viral recombination during productive replication. Defective EBV strains, with the EBNA2 gene deleted, occur naturally in the oropharynxes of 15% of healthy virus carriers and in over 90% of patients with the epithelial lesion oral hairy leukoplakia (12, 24, 26, 27). This wart-like lesion on the lateral borders of the tongue, which often develops in human immunodeficiency virus-infected individuals, has been shown to be a focus of EBV replication (14) and is therefore a likely site for the emergence of viral variants. Although it is not known if such defective EBV strains are common in New Guinea, a related premalignant oral leukoplakia, associated with habitual chewing of betel nut quid, occurs frequently in New Guinea (15) and recent studies in our laboratory have found that EBV is often associated with this condition (unpublished observations). Interestingly, EBNA2-deletion mutants, which differ from prototypic EBV by their inability to immortalize B lymphocytes (19), have been shown to readily recombine with transforming virus in vitro (11, 25, 29). Since coinfection with both EBV types has been documented in New Guinea (2), we presume that the unusual genotype of the MK strain is the result of an intertypic recombination event, perhaps between an EBV-2 strain with EBNA2 deleted and an EBV-1 strain bearing EBNA2. Future EBV typing studies should examine more than one latent antigen region to reveal the frequency and variety of intertypic recombinants in wild-type EBV strains from different geographic regions and associated with different clinical conditions.

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